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14. ABSTRACT
This research combines behavioral, electrophysiological, and molecular approaches to elucidate the cellular basis for learning impairment in a mouse model for fragile X syndrome (FXS), using olfactory learning as a model system. We hypothesize that FMRP, the protein missing in FXS, participates in two aspects of circuit function that are critical to learning: synaptic plasticity and the generation and survival of new neurons in the adult brain. Efforts in the first year of support were directed to laying the groundwork for the experimental studies of the project, which we expect to complete in years two and three. Colonies of mice with the needed mutations have been established and all of the personnel for the studies are in place and trained. Significant advances have been made in the establishment of behavioral paradigms to test both hippocampal -dependent and -independent forms of olfactory learning. Experimental paradigms have also been refined for the study of neurogenesis in the olfactory bulb, glutamate receptor expression as it relates to olfactory learning in olfactory cortex and hippocampus, and the effects of age on glutamate receptor expression. These studies are likely to advance our understanding of intellectual disability and autism in addition to the specific condition of fragile X syndrome. This knowledge will be necessary for the development of rational strategies for prevention and treatment of cognitive impairments from a variety of causes.

15. SUBJECT TERMS
Fragile X syndrome, synaptic plasticity, long-term potentiation, glutamate receptors, neurogenesis, olfactory learning

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INTRODUCTION

Fragile X syndrome is the most common inherited form of intellectual disability. The disorder is caused by mutation in the *FMR1* gene that transcriptionally silences the gene and results in lack of production of the encoded protein, FMRP. A mouse model of the human syndrome (the *Fmr1*-KO mouse) reproduces the protein (FMRP) deficiency and exhibits abnormalities of synaptic structure and function as well as learning impairments. This research combines behavioral, electrophysiological, and molecular approaches to elucidate the cellular basis for learning impairment in *Fmr1*-KO mice using olfactory learning as a model system. We hypothesize that FMRP, the protein missing in FXS, participates in two aspects of circuit function that are critical to learning: synaptic plasticity and the generation and survival of new neurons in the adult brain. First, we use molecular tools to study the cellular basis for neurogenesis deficits in adult *Fmr1*-KO mice and the signaling pathways by which FMRP regulates neurogenesis in both the olfactory bulb and the hippocampus of the adult brain. We test the hypothesis that selective down-regulation of the pro-apoptotic gene, *Bax*, in neurogenic niches will reverse neurogenic deficits in *Fmr1*-KO mice. Second, electrophysiological, biochemical, and pharmacological methods are used to characterize synaptic dysfunction in the olfactory-hippocampal circuit in *Fmr1*-KO mice. We hypothesize that absence of FMRP disrupts trafficking of NMDA receptors to synapses, resulting in impairments in NMDA-dependent synaptic plasticity mechanisms such as long-term potentiation (LTP). Third, using behavioral analyses, we attempt to determine the contributions of neurogenic and synaptic dysfunction to learning impairments in *Fmr1*-KO mice. Finally, we test the hypothesis that impaired neurogenesis underlies the learning impairment in *Fmr1*-KO mice by experimentally stimulating neurogenesis in the mice. In summary, this project will exploit the advantages of the olfactory system to study the cellular basis for learning impairment in a mouse model for fragile X syndrome. This is likely to also have impact on our understanding of mental retardation in general as well as learning disabilities in other autism spectrum disorders. This knowledge will be necessary for the development of rational strategies for prevention and treatment of cognitive impairments from a variety of causes.

BODY

Cellular Basis for Learning Impairment in Fragile X Syndrome

1. Regulatory approvals (re: SOW task 1) were obtained from the UIC IACUC (Mar. 26, 2012, in anticipation of award) and the DoD ACURO (Apr. 23, 2012).

2. Recruitment of research staff: Mr. Andrew Widmer, GRA; Ms. Samantha Keil, technician. Mr Widmer is a combined MD/PhD student in the UIC Medical Scientist Training Program. He has a Bachelor's degree in Biology from Carleton College and research training at the Rockefeller University. Mr. Widmer will be completing the electrophysiological studies of Specific Aims 2 and 3 in years 2 and 3 of this project as part of his PhD dissertation work (re: SOW tasks 3a, 3b, 3c). Ms. Keil was recruited from Augustana College with Bachelor's degrees in Biology and Neuroscience. She will be completing the behavioral studies of Specific Aims 2 and 3 (re: SOW tasks 4a, 4b, 4c). She is also responsible for genotyping all *Fmr1*-KO mice in the colony.

3. Re-derivation of *Fmr1* mutation in C57Bl/6 mice and building up of colony (re: SOW tasks 2-4). The *Fmr1*-KO mouse colony was established with stock derived from frozen embryos and purchased from Jackson Laboratories in July 2012, in anticipation of this award. The initial shipment of *Fmr1*-KO mice was expanded by breeding and interbreeding with C57Bl/6 mice to allow production of littermate progeny of both *Fmr1*-KO and WT genotypes. We now have a thriving colony, in production of *Fmr1*-KO mice and WT littermate controls for experiments.

4. We have started preliminary immunohistochemical analysis of proliferation history of neural progenitor cells in the subventricular zone of *Fmr1*-KO mice using the proposed antibodies and reagents (re: SOW task 2a). Mice were doubly injected with iododeoxyuridine (IdU) and chlorodeoxyuridine (CldU). Brains were sectioned by microtome and sections were subject to immunohistochemical analysis (Figure 1).
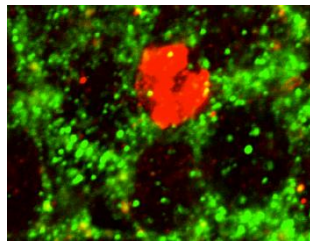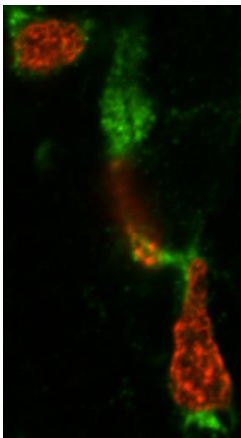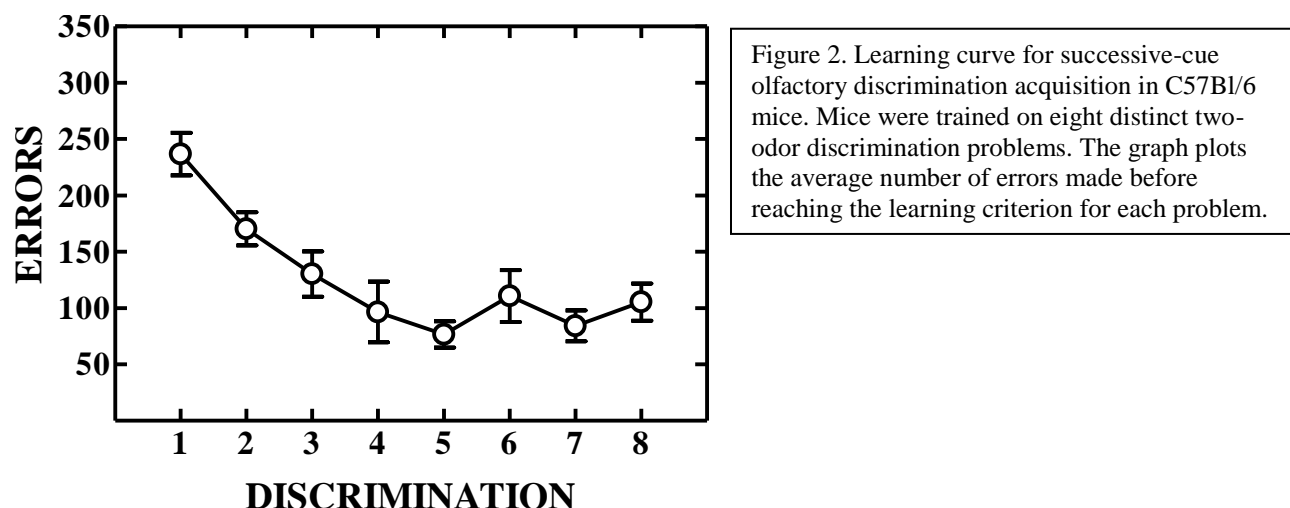

Figure 1. Representative image of an iododeoxyuridine+ (IdU+) cell (Right) and chlorodeoxyuridine+ and β-tubulin+ (CldU+Tuj-1+) cells (left) in the subventricular zone of *Fmr1*-KO mice.

5. We established Baxlox/lox and NestinCreERT2/+ colonies. Both colonies were re-derived and expanded and the generation of the double transgenic nestin-CreERT2/Baxlox/lox has started. These mice are an upgraded substitute to our original plan to enhance neurogenesis with retroviral vectors expressing Bax (re: SOW task 2c). These mice will allow us the generation of an *Fmr1*-KO/nestin-CreERT2/Baxlox/lox mice, that will constitute a reproducible mouse line in which neurogenesis is up-regulated.

6. Parametric development of successive-cue olfactory discrimination task (re: SOW task 4b). Experiment 4b calls for the testing of *Fmr1*-KO and WT control mice in a non-hippocampal dependent olfactory discrimination task. We have run several pilot experiments during the first year of this project to optimize a successive-cue discrimination paradigm to compare learning rates in mutant and control mice. The best paradigm (tested with C57Bl/6J control mice) used a series of eight separate odor discrimination problems as follows: Mice (3 months old) were trained on each discrimination problem in 100-trial sessions in which each trial involved presentation of one of the discriminative odors to both sniff ports in our standard discrimination chamber. Each trial was signaled by the extinguishing of a lamp and the presentation of the odor for 5 sec. A nose poke in either port within the 5 sec presentation period constituted a "GO" response; no response in the same period constituted a "NO-GO" response. GO responses to the positive cue (S+) or NO-GO responses to the negative cue (S-) are scored as correct; GO responses to S- or NO-GO responses to S+ are scored as errors. Only Go responses to S+ are rewarded with a drop (10 μl) of water. The inter-

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trial interval (ITI) after correct responses was 10 sec and the ITI after errors was 30 sec. Each of sixteen mice was trained to criterion performance of eight consecutive correct responses within a single 100-trial training session. (Note that, since no more than three S+ or S- trials can occur consecutively, this criterion requires the mouse to respond correctly to at least two S+ and S- within the sequence of eight consecutive correct responses. The probability of 8 consecutive correct responses by chance is 1/256 or ~0.4%.) Sessions are repeated on subsequent days if criterion performance is not met. The results are presented in Figure 2. All mice learned all of the eight discrimination problems in 10-17 training sessions. The second block of four discrimination problems were learned significantly faster than the first block, evidencing acquisition of learning set behavior. We believe that this paradigm is adequate for a fair comparison of learning rates in *Fmr1*-KO and WT mice and will be very useful, in conjunction with the simultaneous-cue (hippocampal-dependent) learning task for assessing the role of neurogenesis in the hippocampus and olfactory bulb on learning in *Fmr1*-KO mice (SOW task 4c). The new successive-cue paradigm may also prove diagnostic for analysis of response biases using signal detection theory in mutant mice since responses can be categorized into Hits and Misses (S+) and False Alarms and Correct Rejections (S-).

Figure 2. Learning curve for successive-cue olfactory discrimination acquisition in C57Bl/6 mice. Mice were trained on eight distinct two-odor discrimination problems. The graph plots the average number of errors made before reaching the learning criterion for each problem.

7. Analysis of glutamate receptor in olfactory cortex: changes with olfactory discrimination learning (re: SOW task 3a). The goal of experiments in 3a is to understand how absence of FMRP affects trafficking of glutamate (NMDA and AMPA) receptors to synapses in hippocampus and olfactory cortex. We have obtained preliminary evidence that olfactory discrimination training selectively up-regulates NMDA receptor (GluN1) expression in hippocampus of WT mice. The experiments were run by undergraduate research assistant, Jennifer Sotto, in collaboration with Drs. Neil Smalheiser and Giovanni Lugli. Mice were trained to criterion (70% correct in a block of 20 trials) on each of four different two-odor discriminations in the simultaneous-cue discrimination task. Three separate sets of controls were run: (a) home cage controls were never trained at all; (b) nose poke controls were trained to nose poke in the same chamber but were not exposed to odors; (c) odor exposed controls were trained to nose poke with the same odors presented as in the discrimination-trained mice, but the odors had no reward significance. The preliminary data indicate that odor discrimination training resulted in higher expression of GluN1, but not GluA1, in hippocampus. If these results are replicated in further groups of mice (in progress), it may provide an exciting new way to assess the role of FMRP in regulation of expression and trafficking of NMDA receptors to synapses, particularly when animals are challenged with new learning.

8. Completion of study of glutamate receptor changes in olfactory cortex with age in C57Bl/6 mice (re: SOW tasks 3a and 4a): manuscript published (Gocel & Larson, *Front. Aging Neurosci.*, **5**, Article 39, 2013). Parts of Aims 2 and 3 of the present project involve possible age-dependent changes in the role of FMRP in synaptic plasticity, neurogenesis, and learning. We laid the groundwork for studies of FMRP-dependent changes in synaptic expression of AMPA receptors across the lifespan with an electrophysiological analysis of AMPA receptor expression in olfactory cortex synapses in young and old (WT) mice. We found a significant decrease in miniature excitatory postsynaptic current amplitude in aged mice.

KEY RESEARCH ACCOMPLISHMENTS

- Developed a production colony of *Fmr1*-KO and control (WT) mice for experiments.
- Verified detection of neural progenitor cells in subventricular zone of *Fmr1*-KO mice.
- Established colonies of Baxlox and NestinCreERT2/+ mice for neurogenesis experiments.
- Established an efficient paradigm for comparison of hippocampal-independent olfactory learning in *Fmr1*-KO and WT mice.
- Obtained preliminary evidence that NMDA receptor expression is enhanced in hippocampus of mice after extensive olfactory learning.
- Demonstrated that NMDA receptor trafficking to synapses is impaired in olfactory cortex of aging mice.

REPORTABLE OUTCOMES

1. Gocel, J. & Larson, J. Evidence for loss of synaptic AMPA receptors in anterior piriform cortex of aged mice. *Frontiers in Aging Neuroscience,* **5** (2013) Article 39.

CONCLUSIONS

Efforts in the first year of support were mainly directed toward laying the groundwork for the experimental studies of the project, which we expect to complete in years two and three. Colonies of mice with the needed mutations have been established and all of the personnel for the studies are in place and trained. Significant advances have been made in the establishment of behavioral paradigms to test both hippocampal -dependent and -independent forms of olfactory learning. This is important because the electrophysiological impairments of synaptic plasticity that we have observed in the fragile X mouse model occur in olfactory cortex but not hippocampus. Experimental paradigms have also been refined for the study of neurogenesis in the olfactory bulb, glutamate receptor expression as it relates to olfactory learning in olfactory cortex and hippocampus, and the effects of aging on glutamate receptor expression. These studies will allow us to establish a cellular basis for certain aspects of the cognitive disability that characterizes the human fragile X syndrome.

REFERENCES

1. Gocel, J. & Larson, J. Evidence for loss of synaptic AMPA receptors in anterior piriform cortex of aged mice. *Frontiers in Aging Neuroscience,* **5**, article 39 (2013).

Evidence for loss of synaptic AMPA receptors in anterior piriform cortex of aged mice

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It has been suggested that age-related impairments in learning and memory may be due to age-related deficits in long-term potentiation of glutamatergic synaptic transmission. For example, olfactory discrimination learning is significantly affected by aging in mice and this may be due, in part, to diminished synaptic plasticity in piriform cortex. In the present study, we tested for alterations in electrophysiological properties and synaptic transmission in this simple cortical network. Whole-cell recordings were made from principal neurons in slices of anterior piriform cortex from young (3–6 months old) and old (24–28 months) C57Bl/6 mice. Miniature excitatory postsynaptic currents (mEPSCs) mediated by AMPA receptors were collected from cells in presence of tetrodotoxin (TTX) and held at −80 mV in voltage-clamp. Amplitudes of mEPSCs were significantly reduced in aged mice, suggesting that synaptic AMPA receptor expression is decreased during aging. In a second set of experiments, spontaneous excitatory postsynaptic currents (s/mEPSCs) were recorded in slices from different cohorts of young and old mice, in the absence of TTX. These currents resembled mEPSCs and were similarly reduced in amplitude in old mice. The results represent the first electrophysiological evidence for age-related declines in glutamatergic synaptic function in the mammalian olfactory system.

Keywords: piriform cortex, mEPSC, LTP, AMPA, aging, mouse, olfaction

INTRODUCTION

Changes in the nervous system with aging are profound and mysterious. The brain exhibits subtle alterations in cellular morphology, synaptic structure, gene expression patterns, and electrophysiological characteristics as it ages; less subtle, perhaps, are the sensory, motor, and cognitive declines that accompany the aging process. Understanding the relationships between neurobiological changes and functional outcomes is one of the fundamental challenges of aging neuroscience. Considerable progress has been made in correlating age-dependent changes in hippocampal circuitry to spatial learning and memory deficits in aging animals. Two general principles have emerged from studies of hippocampal long-term potentiation (LTP) in aged animals (Burke and Barnes, 2010): first, age-dependent effects on synaptic function are regionally heterogeneous. For example, synaptic density declines in the dentate gyrus, but not field CA1, of old rats. Electrophysiological studies using minimal stimulation suggest that "basal" synaptic potency, the average size of the postsynaptic response to a presynaptic release event (Stevens and Wang, 1994), declines with extreme age in CA1 but not dentate. Second, LTP induction mechanisms are typically only impaired when stimulation is close to the induction threshold; LTP expression, provided that induction conditions are suprathreshold, appears normal (Burke and Barnes, 2010).

The olfactory system has a number of advantages for neurobiological studies of sensory and cognitive functions in aging. Olfactory discrimination ability declines markedly in human aging (Doty et al., 1984; Cain and Stevens, 1989) and appears to be particularly vulnerable to age-related neurodegenerative disease

(Serby et al., 1985; Kesslak et al., 1988). Olfactory dysfunction has also been reported for aging rodents (Roman et al., 1996; Frick et al., 2000; Schoenbaum et al., 2002; Enwere et al., 2004; Prediger et al., 2005; LaSarge et al., 2007). For instance, a recent study from our laboratory found that old mice took more trials to learn two-odor discrimination problems for positive reinforcement and failed to show improvement across multiple discrimination problems when compared to young mice (Patel and Larson, 2009). However, the neurobiological bases for these deficits are largely unexplored.

The primary olfactory (piriform) cortex receives monosynaptic input from the mitral (and tufted) cells of the olfactory bulb, which themselves receive monosynaptic input from primary sensory neurons in the olfactory epithelium. Layer II pyramidal neurons of piriform cortex appear to be situated to form combinatorial representations of the different olfactory bulb glomeruli that respond to distinct molecular features of the chemicals comprising an odor (Wilson and Sullivan, 2011). Piriform neurons project monosynaptically to the lateral entorhinal cortex, providing olfactory input to the hippocampal formation, as well as to parts of the amygdalar complex and the prefrontal cortex (Shipley et al., 1995). Both the afferent synapses made by the mitral cells onto layer II pyramidal cells and the associational feedback system generated by neighboring pyramidal cells are glutamatergic and exhibit LTP (Jung et al., 1990a,b; Kanter and Haberly, 1990), possibly to strengthen representations of learned odors or to participate in odor–reward associations. The present studies were directed to test for synaptic functional changes in anterior piriform cortex (APC) of aged mice. The results provide the first

direct evidence that aging results in decreases in synaptic currents mediated by AMPA-type glutamate receptors in a simple cortical network.

MATERIALS AND METHODS
ANIMALS
C57Bl/6J mice were bred in the Psychiatric Institute vivarium from breeding stock obtained from Jackson Laboratories (Bar Harbor, ME, USA). Mice were weaned at 21 days of age and males were separated and housed in groups of two to four per cage until sacrificed for experiments. Food and water were available *ad libitum* and routine veterinary visits ensured that animals were in good health prior to experimentation. Any animals that displayed physical ailments or lethargy were excluded from this study. Electrophysiological experiments were conducted on brain slices obtained from mice aged 3–6 months ("young"; $n = 16$) or 24–28 months ("old"; $n = 12$). All procedures were in accordance with NIH guidelines and protocols were approved by the Animal Care Committee of the University of Illinois at Chicago (UIC ACC #09-232).

ELECTROPHYSIOLOGY
In vitro slice preparation and electrophysiology
Parasagittal slices (300 μm) of APC were prepared in the usual method. Mice were decapitated and brains removed in oxygenated artificial cerebral spinal fluid (aCSF) containing (in mM): NaCl (120), KCl (3.1), K_2HPO_4 (1.25), $NaHCO_3$ (26), dextrose (5.0), L-ascorbate (2), $MgCl_2$ (1.0), $CaCl_2$ (2.0) at ~4°C. The brain was then sectioned into blocks (2–3 mm thick), mounted on a cutting stage, and sliced on a vibrating cutter (Vibratome, St. Louis, MO, USA). The slices were incubated at 32°C for 1 h and then allowed to cool to room temperature (~25–27°C). Slices were then transferred to a submerged recording chamber and perfused with aCSF. Recordings were obtained from principal cells in layer II at room temperature, as described previously (Gocel and Larson, 2012). Patch electrodes (1.8–3 MΩ) contained (in mM): cesium methanesulfonate (145), $MgCl_2$ (1), HEPES (10), BAPTA (1.1), MgATP (5), and phosphocreatine (20) adjusted to pH 7.2 with CsOH, 290 mOsm. Cells were visualized with differential interference contrast (DIC) optics on a Nikon Eclipse E600FN "PhysioStation" (Nikon, Melville, NY, USA). Twisted bipolar electrodes (custom made) with a tip diameter ~50 μm were positioned in layer Ia and Ib 150–200 μm rostral from the recorded cell. Stimulation (0.1 ms pulses 1–200 μA) in these layers activate afferent lateral olfactory tract (LOT) and intrinsic association (ASSN) fibers synapsing onto principal cells in layer II. Responses at ASSN synapses display paired-pulse depression whereas stimulation of afferent LOT fibers in layer Ia exhibit facilitation (Bower and Haberly, 1986). Therefore, stimulation of ASSN fibers in layer Ib was confirmed after obtaining whole-cell recording by non-facilitating responses to paired-pulse stimulation at a 200 ms inter-pulse interval (IPI). Evoked excitatory postsynaptic currents (EPSCs), miniature EPSCs (mEPSCs) and spontaneous EPSCs (s/mEPSCs) were recorded with an Axopatch-1D amplifier and pClamp software (Molecular Devices, Sunnyvale, CA, USA), filtered at 1 kHz, digitized at 10 kHz, and stored on the computer hard drive. Cells were immediately rejected if series resistance exceeded 15 MΩ upon obtaining whole-cell recording configuration. Series and whole-cell capacitance compensation and junction potential correction were not used.

PHARMACOLOGY
All drugs and chemicals were applied via the perfusate by a solenoid-controlled gravity-feed system (ValveLink 8, AutoMate Scientific, Inc., Berkeley, CA, USA). The rate of flow of all drug perfusates was equilibrated to 2 mL/min prior to the inception of experimentation. $GABA_A$ mediated transmission was blocked by 25 μM 1(S),9(R)-(−)-bicuculline methiodide (BMI) in all experiments in order to isolate postsynaptic excitatory currents. The NMDA receptor antagonist, 3-[(R)-2-carboxypiperazin-4-yl]-prop-2-enyl-1-phosphonic acid (CPP, 20 μM)) was used to isolate AMPA receptor-mediated currents. 1 μM tetrodotoxin (TTX) was added to the perfusate in order to eliminate spontaneous action potential-dependent events. Some experiments used 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) to block AMPA receptors (20 μM). All drugs were taken from stock solutions dissolved in H_2O and diluted in aCSF as needed.

EVOKED SYNAPTIC CURRENTS
In experiments using synaptic stimulation under voltage-clamp, the following stimulation protocol was used: each trial began with a baseline recording period of 300 ms, followed by paired-pulse stimulation of the LOT (50 ms IPI), a one-second delay, and paired-pulse stimulation of the ASSN fibers (50 ms IPI). Stimulation current levels were set to yield a reliable response with the least amount of stimulation in order to avoid polysynaptic activity or synaptic population crosstalk. Stimulation intensities were the following: LOT: 3–6 months, 40.61 ± 12.12 μA; 24–28 months, 120.00 ± 47.97 μA and ASSN: 3–6 months, 26.17 ± 3.02 μA, 24–28 months, 60.89 ± 11.17 μA. Any recordings demonstrating polysynaptic activity were excluded from analysis. Measurements of averaged (50–100 trials) current attributes were performed in Clampfit.

mEPSC AND s/mEPSC ANALYSIS
For analysis of mEPSCs, slices were perfused with TTX (1 μM) until whole-cell currents evoked by LOT and ASSN fiber stimulation were abolished. Cells were maintained at a holding potential of −80 mV throughout recordings and bicuculline and CPP were used to prevent activation of $GABA_A$ and NMDA receptors, respectively. Spontaneous synaptic currents were recorded for 200 s of continuous recording from every cell. mEPSCs were identified as follows: A single 50 ms variable amplitude template was constructed (Clements and Bekkers, 1997) from >20 visually-identified events in a randomly-selected cell and served as a search criterion for collecting and aligning mEPSCs and s/mEPSCs for all cells. Cells were excluded from analysis if the baseline drifted more than 50 pA or if access resistance changed more than 20% throughout the 200 s of continuous recording. All compound events were excluded from analysis. Measurements of the current waveforms (kinetics) were obtained from an average of all events (>100) that met analysis criteria in each cell. Decay time constants were fit to averaged mEPSCs with the standard exponential equation

$I(t) = I \times \exp(-t/\tau)$, where I is the peak amplitude and τ is a the decay time constant.

Spontaneous EPSCs (s/mEPSCs) were recorded and quantified as for mEPSCs, except that TTX was not used.

HISTOLOGY

Some cells used for electrophysiology were also filled with a 4% Lucifer yellow solution passively during the recordings. Brain sections were then immediately fixed in 4% paraformaldehyde overnight, whole mounted on glass slides and coverslipped. Additional physiological slices fixed in 4% paraformaldehyde were cryoprotected in 30% sucrose in PBS. These slices were then resectioned at 30 μm, processed, and stained with 0.1% cresyl violet. Filled cells were visualized under a fluorescein isothiocyanate (FITC) filter and stained slices were visualized under bright field illumination on an Axioskop 2 microscope. Images were acquired through Axiovision software (Zeiss, Thornwood, NY, USA) and edited in GNU image manipulation program (GIMP, open source).
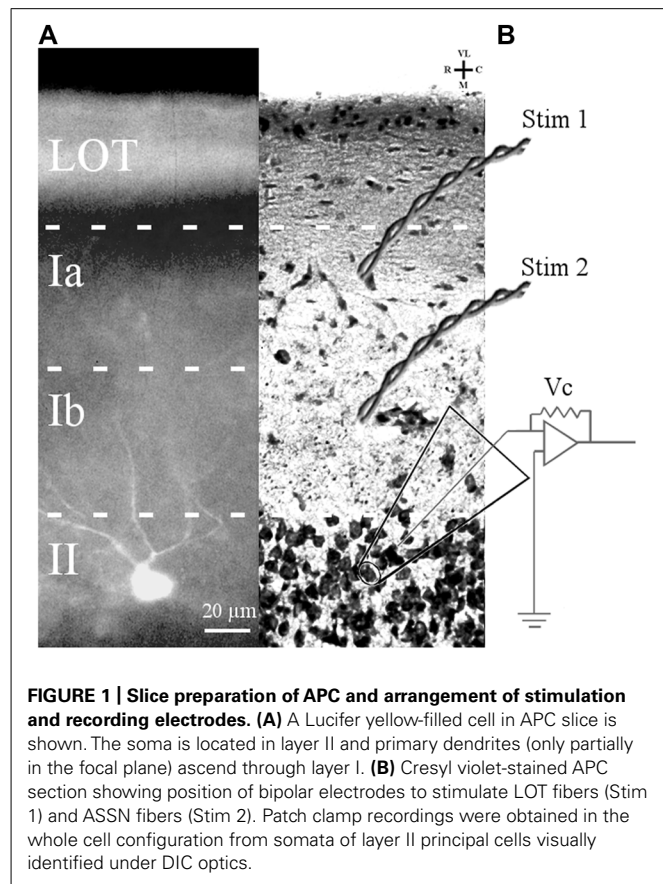
STATISTICS

All data are based on cells as individual sample units and are presented as means ± SEM. Statistical differences between two groups were evaluated using Student's unpaired *t*-test. Analysis of variance was used for comparisons involving more than two groups.

RESULTS

A subset of the cells chosen for electrophysiological study were filled with Lucifer yellow to confirm that they were layer II pyramidal cells (**Figure 1**). Filled cells from mice at all ages showed multiple, spiny apical dendrites which projected toward the pial surface. Other slices stained with cresyl violet were examined for cytoarchitecture. There were no obvious differences in soma morphology, cortical lamination, or cell density of APC of 3–6 months old and 24–28 months old mice.

AMPA RECEPTOR-MEDIATED mEPSCs ARE SMALLER IN THE AGED MOUSE

As described previously (Gocel and Larson, 2012), superficial pyramidal (SP) neurons held at −80 mV under voltage clamp in the presence of BMI, CPP, and TTX showed spontaneous inward currents with the characteristics of mEPSCs, in slices from both young and old mice (**Figure 2A**). These events were abolished by perfusion with CNQX, confirming that they were mediated by AMPA receptors (data not shown). Synaptic events were collected from continuous 200 s recording epochs, aligned, averaged, and analyzed. Amplitude distributions were positively skewed in both young (**Figure 2B**) and old mice (**Figure 2C**). The mean amplitudes of mEPSCs recorded in slices from old mice were significantly smaller than those from young mice (**Figure 2D**). Since the amplitude distributions were skewed, the median mEPSC amplitude was also calculated for each cell and compared between age groups. These measures were also significantly smaller in the aged mice (young: 12.31 ± 0.51 pA; old: 9.95 ± 0.53 pA; $p < 0.01$). The average frequency of detected mEPSCs did not significantly differ between age groups (**Figure 2E**). Cumulative

FIGURE 1 | Slice preparation of APC and arrangement of stimulation and recording electrodes. (A) A Lucifer yellow-filled cell in APC slice is shown. The soma is located in layer II and primary dendrites (only partially in the focal plane) ascend through layer I. **(B)** Cresyl violet-stained APC section showing position of bipolar electrodes to stimulate LOT fibers (Stim 1) and ASSN fibers (Stim 2). Patch clamp recordings were obtained in the whole cell configuration from somata of layer II principal cells visually identified under DIC optics.

amplitude distributions (**Figure 2F**) also illustrate the shift in amplitude toward smaller mEPSCs in the neurons from old mice. Possible age-related changes in the kinetics of AMPA receptors mediating mEPSCs were calculated by fitting a single exponential function to the decay phase of averaged mEPSCs in each cell. The decay time constants tended to be longer in cells from old mice, although this difference only approached statistical significance (young: 4.22 ± 0.16 ms, $n = 24$; old: 4.72 ± 0.18 ms, $n = 14$; $t_{36} = 1.95$, $p = 0.06$).

PAIRED-PULSE RESPONSES ARE UNAFFECTED BY AGING

The vast majority of glutamatergic synapses on SP neurons in APC are generated by either afferents from the olfactory bulb (LOT), terminating in the outer molecular layer (Ia) or the intrinsic associational (ASSN) system generated by the SP neurons themselves and terminating in the inner molecular layer (Ib). To test whether or not changes in the potency of individual synapses (mEPSCs) with aging were accompanied by presynaptic changes in release characteristics at either or both of these systems, paired-pulse stimulation (50 ms IPI) was applied to each pathway while recording from cells held at −80 mV in slices from young and old mice. Cells were only included in analysis if responses were present from both synaptic pathways. All traces collected for a given cell which met criteria were averaged and the percent potentiation or depression of the second response was calculated relative to that of the first response (**Figure 3**). The robust facilitation of responses to LOT stimulation was not significantly altered by
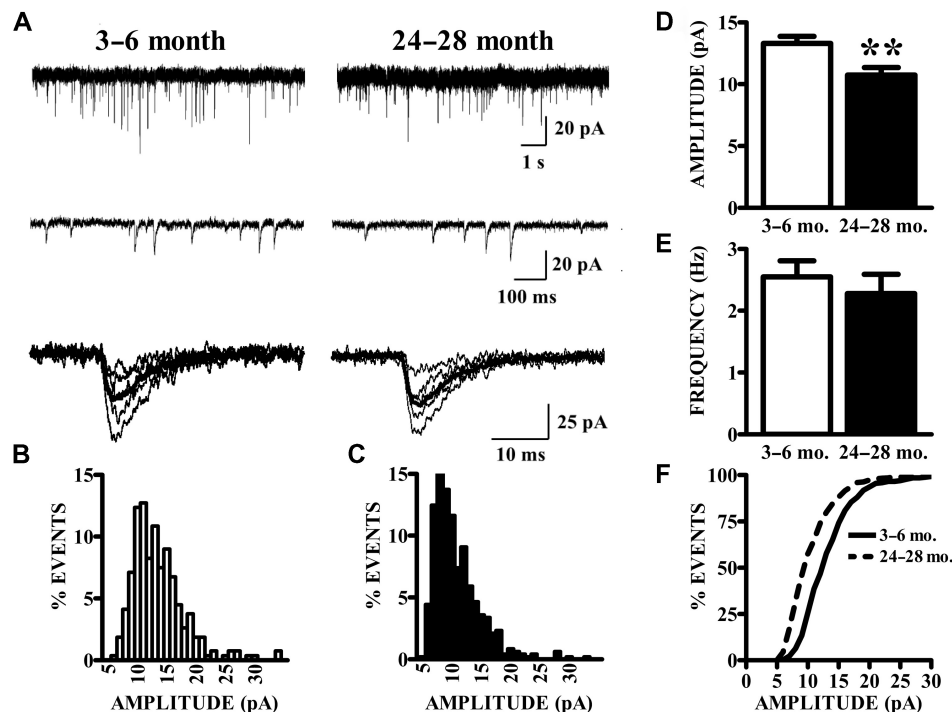
FIGURE 2 | AMPA receptor-mediated currents at single synapses are reduced in aged mice. (A) mEPSCs were collected in the presence of TTX. Top raw traces were extracted from 200 s recording epochs from which spontaneous currents were collected. Middle traces are expanded sections of the top traces; a template algorithm was used to search for individual currents. Bottom traces are selected individual mEPSCs aligned according to onset of the current. **(B,C)** All currents collected during the 200 s epochs were binned according to amplitude. As demonstrated by these representative histograms, AMPA mEPSC amplitude histograms in old (24–28 months) animals **(C)** demonstrated a leftward shift in distribution as compared to young (3–6 months) animals **(B)**. **(D)** Mean amplitudes (shown) of AMPA mEPSCs were significantly decreased in old relative to young mice ($t_{36} = 2.87$, $p < 0.01$). Comparison of both age groups according to the median amplitude (not shown) also demonstrated a reduction. **(E)** The frequency of mEPSCs was comparable between age groups. Frequency was determined by counting the events that occurred during the entire 200-s recording epochs. **(F)** Cumulative distributions illustrate the shift in the mEPSC amplitudes.

age. ASSN responses showed neither facilitation nor depression at the interval tested; normalized responses were also unaffected by age.
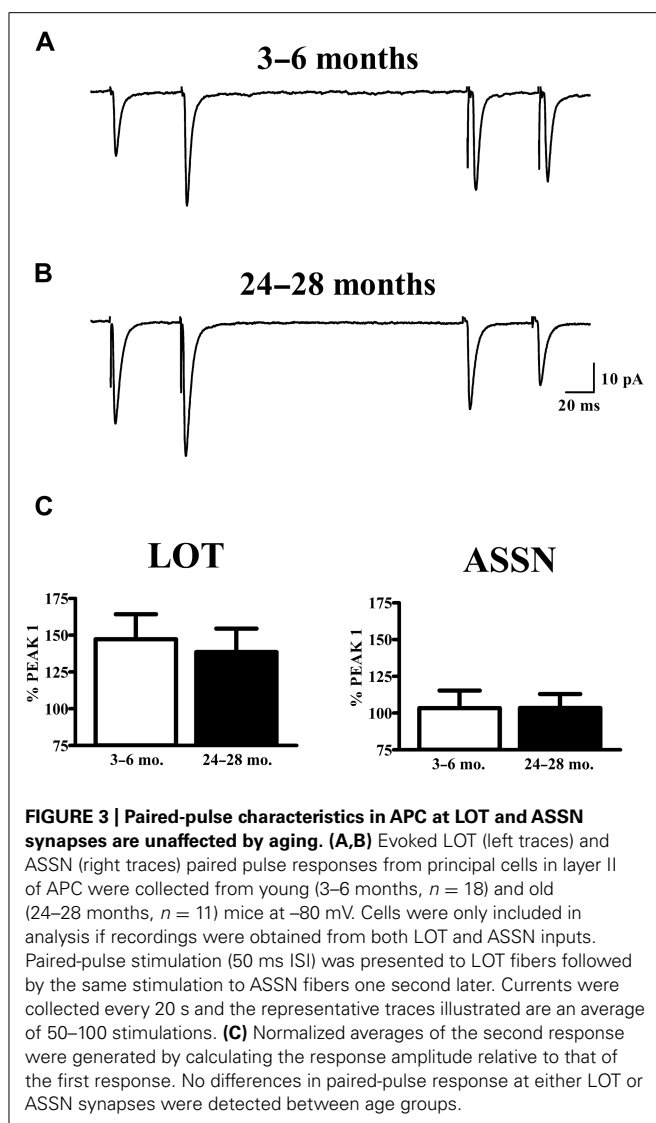
SPONTANEOUS SYNAPTIC CURRENTS (s/mEPSCs) ARE REDUCED IN THE AGED MOUSE

Spontaneous currents in the absence of stimulation were obtained from the same cells in which evoked paired-pulse responses were obtained. Experiments were performed in the absence of TTX in order to obtain evoked responses; therefore, the spontaneous currents collected are presumed to be a mixture of action potential-dependent (sEPSCs) and independent (mEPSCs) events. The manner of analysis was identical to the method of analyzing mEPSC data. Aged animals exhibited a significant reduction in mean (**Figure 4**) and median (young: 12.78 ± 0.53 pA; old: 10.69 ± 0.41 pA; $p < 0.01$) s/mEPSC amplitude. The similar size of mEPSCs recorded in the presence of TTX (**Figure 2**) and s/mEPSCs recorded in the absence of TTX (**Figure 4**) suggests either (1) that action potential-dependent and -independent release events evoke similar postsynaptic responses or (2) that the action potential-dependent (TTX-sensitive) events are a small fraction of spontaneous release events in these cells. Frequency distributions of s/mEPSC amplitudes also did not reveal any

obvious differences with those of mEPSCs. In any case, it bears noting that the s/mEPSC (**Figure 4**) and mEPSC (**Figure 2**) recordings were made from completely different sets of young and old animals.

DISCUSSION

There is compelling evidence that olfaction-mediated sensory and cognitive functions decline with aging in humans (Doty et al., 1984; Cain and Stevens, 1989; Wysocki and Gilbert, 1989; Larsson et al., 2000, 2006; Gilbert et al., 2008) and experimental animals (Roman et al., 1996; Frick et al., 2000; Enwere et al., 2004; Prediger et al., 2005; LaSarge et al., 2007; Dardou et al., 2008; Luu et al., 2008; Patel and Larson, 2009). The piriform cortex occupies a strategic position in the neural processing of odors: (i) it is the largest target of efferents from the olfactory bulb (Neville and Haberly, 2004); (ii) its internal wiring suggests a combinatorial mechanism for synthetic integration of odor percepts from multiple chemical constituents that activate distinct odorant receptor proteins and corresponding glomeruli in the olfactory bulb (Haberly, 2001; Franks and Isaacson, 2006; Stettler and Axel, 2009; Wilson and Sullivan, 2011); (iii) odor discrimination and olfactory learning are disrupted by piriform lesions (Staubli et al., 1987); (iv) olfactory training modifies synaptic structure and function in

FIGURE 3 | Paired-pulse characteristics in APC at LOT and ASSN synapses are unaffected by aging. (A,B) Evoked LOT (left traces) and ASSN (right traces) paired pulse responses from principal cells in layer II of APC were collected from young (3–6 months, $n = 18$) and old (24–28 months, $n = 11$) mice at –80 mV. Cells were only included in analysis if recordings were obtained from both LOT and ASSN inputs. Paired-pulse stimulation (50 ms ISI) was presented to LOT fibers followed by the same stimulation to ASSN fibers one second later. Currents were collected every 20 s and the representative traces illustrated are an average of 50–100 stimulations. **(C)** Normalized averages of the second response were generated by calculating the response amplitude relative to that of the first response. No differences in paired-pulse response at either LOT or ASSN synapses were detected between age groups.

FIGURE 4 | AMPA s/mEPSC amplitudes are reduced in APC of old mice. (A,B) Raw traces illustrated include events collected during recording periods where no stimulation was presented in the same cells shown in **Figure 3**. Spontaneous currents (s/mEPSCs) in these recordings are presumed to emanate from action potential-mediated and -independent events since TTX was not present in the perfusate. **(C)** The mean amplitude of s/mEPSCs was calculated and averaged across cells. Events collected from aged (24–28 months, $n = 11$) mice demonstrated a decrease in amplitude compared to those from young (3–6 months, $n = 18$) mice ($t_{27} = 2.48$, $p < 0.05$).

piriform cortex (Barkai and Saar, 2001); and (v) it is the primary pathway by which olfactory information reaches the hippocampus, amygdala, and prefrontal cortex (Shipley et al., 1995). However, this structure has received very little attention in neurobiological studies of aging. The present study represents an initial step in a comprehensive analysis of the brain substrate for olfactory dysfunction in aging.

We recorded from principal neurons in primary olfactory cortex from young adult and aged mice. The main finding of the experiments described here is a decrease in the amplitude of synaptic currents mediated by AMPA receptors on these cells in aged mice. TTX-resistant spontaneous synaptic currents (mEPSCs) are thought to be evoked by stochastic release of single glutamate quanta at individual synapses. Assuming that synaptic vesicles are the physical basis for quantal release, there are two main ways to alter quantal size: a change in glutamate loading of vesicles or a change in postsynaptic receptors in the synaptic zone. There is little precedent for changes in vesicle loading but considerable evidence that postsynaptic AMPA receptor numbers can be
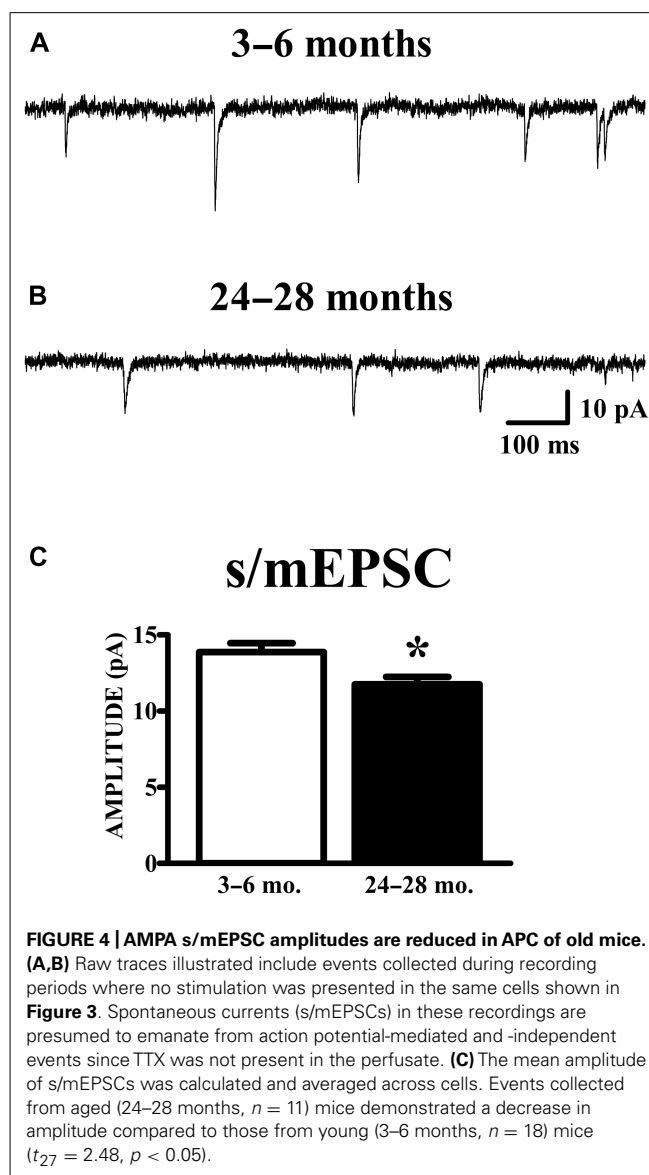
altered in an experience-dependent manner (Lynch and Baudry, 1984; Bredt and Nicoll, 2003) or in certain disease models (Li et al., 2002). Therefore the most parsimonious interpretation of a decrease in mEPSC size is a reduction in the number of functional AMPA receptors activated by synaptic glutamate release in the aged mice. In theory, a postsynaptic mechanism could be confirmed or ruled out by measuring NMDA receptor-mediated mEPSCs; however, this was impractical due to the voltage-dependence and slow kinetics of NMDA receptor-mediated currents. On the other hand, there are numerous reports of decreases in AMPA receptor expression, measured by mRNA or protein expression or ligand binding, in various brain regions of aged rodents (Bahr et al., 1992; Magnusson and Cotman, 1993; Nicoletti et al., 1995; Nicolle et al., 1996; Magnusson, 1998; Wenk and Barnes, 2000; Majdi et al., 2009).

It is important to note that spontaneous EPSCs (sEPSCs) were also recorded from APC cells in slices from completely

independent cohorts of young and old mice, in the absence of TTX. The amplitude distributions of these events were almost identical to the events (mEPSCs) recorded in slices exposed to TTX. This suggests that the sEPSCs and mEPSCs are the same events; the lack of TTX sensitivity may be attributed to the high resting membrane potentials and low spontaneous firing of piriform pyramidal cells *in vitro* (Scholfield, 1978; Tseng and Haberly, 1988; Suzuki and Bekkers, 2006). In any case, the smaller amplitude of spontaneously-occurring synaptic AMPA currents in cells from old mice was replicated for both mEPSCs and "sEPSCs," in different cohorts of mice.

The frequency of mEPSCs was not altered in the aged mice, suggesting that either (i) no change in the number of synapses contributing to these synapses with aging or (ii) that any such changes are accompanied by compensatory changes in spontaneous release probability. The synaptic origin of the mEPSCs could not be determined in these experiments. However, it is worth noting that the paired-pulse experiments did not reveal any differences that would be indicative of changes in release probability in either the LOT or ASSN pathways in young versus old mice. It is also worth noting that paired-pulse ratios may not be very sensitive measures of release kinetics.

In toto, the results of the present study point to the conclusion that aging results in a loss of functional AMPA receptors at synapses on layer II pyramidal cells in piriform cortex. These losses may occur at LOT synapses, ASSN synapses, or both. Further studies will be necessary to establish whether these AMPA receptor losses are due to alterations in receptor subunit transcription, translation, trafficking, post-translational modification, or receptor assembly, as well as whether or not synaptic NMDA receptors are similarly effecting by aging. The decrease in synaptic potency with aging observed in the present study is in contrast to studies using minimal stimulation in rat hippocampal formation where the results suggest that dentate synapses increase in potency (larger unitary EPSPs) while CA1 synapses remain unchanged in old age (Barnes and McNaughton, 1980; Foster et al., 1991; Barnes et al., 1992).

A loss of synaptic AMPA receptors would not be expected to be without effect on LTP in the APC. Although the effects of aging on LTP have not been studied in piriform cortex, activation of more synapses with fewer AMPA receptors would be needed to trigger LTP in aged mice, in order to overcome the voltage-dependent block of NMDA receptors during high frequency stimulation. A disturbance of LTP might not be evident with supramaximal stimulation paradigms but may appear with near threshold stimulation, as observed in hippocampal field CA1. In this scenario, enhancement of AMPA receptor function might compensate for loss of synaptic AMPA receptors. Drugs that act in this way are known to facilitate olfactory learning in young animals (Larson et al., 1995) and may alleviate some of the learning deficits shown by aged mice.

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